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Tumor Necrosis Factor-α Cooperates With Receptor Activator of Nuclear Factor κB Ligand in Generation of Osteoclasts in Stromal Cell-depleted Rat Bone Marrow Cell Culture

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A member of the tumor necrosis factor (TNF) family, receptor activator of nuclear factor κB ligand (RANKL); also known as ODF, OPGL, and TRANCE), plays critical roles in osteoclast differentiation and activation in the presence of macrophage colony-stimulating factor (M-CSF). Recently, TNF-α has also been shown to induce the formation of multinucleated osteoclast-like cells (MNCs) in the presence of M-CSF from mouse macrophages. We demonstrated that mononuclear preosteoclast-like cells (POCs) were formed in the presence of conditioned medium of osteoblastic cells in a rat bone marrow culture depleted of stromal cells. Using this culture system, in this study we examined whether TNF-α affects differentiation into POCs from hematopoietic progenitor cells. Human TNF-α (hTNF-α) markedly stimulated the formation of POCs. Moreover, a concentration as low as 0.005 ng/mL of hTNF-α increased the level of mRNA for calcitonin receptor (CTR) and cathepsin-K of POCs. The POCs induced by hTNF-α formed MNCs, which showed dentine-resorbing activity after coculture with primary osteoblasts. Stimulation was observed after 24 h of treatment with hTNF-α only on day 1 or day 2 of the culture. After 24 h of hTNF-α treatment, expression of the receptor activator of nuclear factor κB (RANK) mRNA was markedly increased. The addition of soluble RANKL (sRANKL) to the preformed POCs efficiently induced MNCs. Interestingly, treatment of bone marrow cells with hTNF-α and sRANKL synergistically augmented the formation of MNCs. This formation was abolished by the addition of human osteoprotegerin (hOPG). These results suggest that cooperation of TNF-α and RANKL is important for osteoclastogenesis. (Bone 28: 474–483; 2001) © 2001 by Elsevier Science Inc. All rights reserved.

Key Words: Osteoclast differentiation; Tumor necrosis factor (TNF)-α; Hematopoietic cell; Receptor activator of nuclear factor κB ligand (RANKL); Osteoclast differentiation factor (ODF); Receptor activator of nuclear factor κB (RANK); Osteoprotegerin (OPG).

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Introduction

Osteoclasts are bone-resorptive multinucleated cells crucial to physiological bone remodeling. These cells are derived from hematopoietic stem cells and are formed by fusion of mononuclear osteoclast precursors.[5,28,46] Osteoclasts also function in the pathological bone loss that occurs in association with inflammatory diseases, such as rheumatoid arthritis (RA),[35] periodontal disease,[44] and postmenopausal osteoporosis.[35]

Recently, a critical regulator of osteoclast differentiation and activation was identified. Receptor activator of nuclear factor κB ligand (RANKL); also known as osteoclast differentiation factor [ODF], tumor necrosis factor-related activation-induced cytokine [TRANCE], and osteoprotegerin ligand [OPGL]) is a tumor necrosis factor (TNF)-related cytokine and induces osteoclast differentiation in the presence of macrophage colony-stimulating factor (M-CSF).[2,29,53,55] The activity of RANKL is mediated by its receptor, RANK, which is a TNF receptor-related protein expressed on osteoclast precursors to provide the signals necessary for osteoclast differentiation.[8,14,33,37] The expression of RANKL is also induced by antigen receptor engagement in T cells.[53] Recently, Kong et al.[23] reported that the production of RANKL by activating T cells leads to bone loss and joint destruction in a rat model of adjuvant arthritis. RANKL is thus involved in not only physiological osteoclastogenesis but also pathological bone loss.

Tumor necrosis factor-α (TNF-α), a multifunctional cytokine produced mainly by activated macrophages, has numerous functions. It is a potent bone-resorbing factor and stimulates osteoclastic bone resorption in vitro[30,50] and in vivo.[20,24] It has been reported that TNF-α participates in inflammatory disease involving loss of bone. Also, it is involved in orthopedic implant osteolysis induced by prosthesis-derived wear particles[34] and experimental periodontitis.[3] Accumulation of TNF-α has been detected in patients affected by RA.[42] TNF-α also plays an important role in bone loss associated with postmenopausal osteoporosis.[1,21] Recently, two laboratories have reported that mouse TNF-α, together with M-CSF, induces osteoclast formation in mouse bone marrow culture. Those investigators used bone marrow cells treated with a high concentration of M-CSF having several macrophage phenotypes as osteoclast progenitor cells and showed that the activity of TNF-α is independent of the RANKL-RANK system.[4,22] On the other hand, TNF-α is known to affect the proliferation and differentiation of hemopoietic stem

cells and myeloid progenitor cells along with other cytokines.[7,15,41] Cooperation between granulocyte macrophage-colony stimulating factor (GM-CSF) and TNF-α is crucial for the differentiation of hemopoietic progenitor cells into dendritic cells,[6,45] suggesting that immature hematopoietic cells could be targets for TNF-α for stimulating osteoclastogenesis.

We developed two types of rat bone marrow culture systems in which the process of osteoclast development could be separated. In one of these systems, multinucleated osteoclast-like cells (MNCs) are formed,[25] whereas, in the other, mononuclear preosteoclast-like cells (POCs) are formed from stroma-free cultures in the presence of conditioned medium of osteoblastic cells.[26] POCs have several characteristics of osteoclasts, including the expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR), and Kat1, a rat osteoclast-specific Ag.[27] In previous studies, we demonstrated that interleukin (IL)-10 inhibited the formation of POCs,[54] whereas IL-15 stimulated differentiation into POCs.[38] To investigate the contribution of TNF-α in osteoclast differentiation, we assessed the effects of TNF-α on the formation of POCs in a rat stroma-free bone marrow culture system. We show that hTNF-α enhances the formation of POCs by stimulating the cells in the early phase of the culture. hTNF-α did not induce MNCs, whereas soluble-form RANKL (sRANKL) did induce MNCs in this culture system. Interestingly, we found that treatment with hTNF-α stimulated the expression of RANK mRNA on osteoclast precursor cells, and that TNF-α with sRANKL could synergistically stimulate the formation of MNCs.

Materials and Methods

Materials

Male Sprague-Dawley rats, aged 5–7 weeks, were obtained from Seac Yoshitomi Co. (Fukuoka, Japan). Alpha-minimum essential medium (α-MEM) was purchased from Gibco (Grand Island, NY). Fetal calf serum (FCS) was purchased from BioWhittaker (Walkersville, MD). 1α,25-dihydroxyvitamin D$_3$ [1,25-(OH)$_2$D$_3$] was purchased from Biomol (Plymouth Meeting, PA). Recombinant hTNF-α and rat TNF-α (rTNF-α) were purchased from Boehringer Mannheim (Mannheim, Germany) and Pepro-Tech (London, UK), respectively. Recombinant human sRANKL, hOPG, and human M-CSF were also purchased from Pepro-Tech. The cytochemical staining kit for TRAP staining was obtained from Sigma (St. Louis, MO). [125I]Iodine-labeled salmon calcitonin ([125I]-sCT) was purchased from Amersham (Buckinghamshire, UK) and NR-M2 emulsion, Konicadol X, and Konicafix were obtained from Konica Co. (Tokyo, Japan).

Stroma-free Bone Marrow Cell Cultures

For the formation of POCs, bone marrow cells were isolated from the tibiae and femurs of rats, and nonadherent bone marrow cells from which adherent cells were eliminated using a Sephadex G-10 column were prepared. The cells were then cultured in α-MEM containing 15% FCS in the presence of 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ and 10% (v/v) heat-treated conditioned medium derived from the rat osteoblastic cell line ROS17/2.8 (htROSCM), as described by Kukita et al.[26] Various concentrations of cytokines were added to the culture for specified periods. For temporal treatment with hTNF-α, the culture was replenished daily with fresh medium, hormone, htROSCM, and hTNF-α. Before the addition of new medium, the cells were washed four times by replacing 80% of the culture medium with fresh medium to eliminate the remaining factors. Factors were then added for a given period. After 4 days of culture, the cells were fixed

and then stained with TRAP using a commercial kit or immunostained with the osteoclast-specific monoclonal antibody (MAb) Kat1.[27] In addition, the presence of CTR in some cultures was detected by autoradiography as described elsewhere.[26] In some experiments, nonadherent bone marrow cells were further purified on a Sephadex G-10 column once more to completely eliminate stromal cells. These purified nonadherent bone marrow cells did not form colonies of stromal cells after 14 days of culture. There was no significant difference in the osteoclastogenesis-enhancing activity of hTNF-α regardless of whether purified nonadherent bone marrow cells or nonadherent bone marrow cells were used. Nonadherent bone marrow cells were used to form POCs in all experiments except where indicated.

Immunocytochemistry

Immunostaining with MAb Kat1 was performed as previously described.[27] Briefly, the cells were incubated with MAb Kat1 for 45 min and fixed with 4% paraformaldehyde for 20 min at room temperature. After being blocked with 3% goat serum for 60 min, the cells were incubated with biotinylated anti-mouse immunoglobulin (IgM) as the second antibody for 30 min and then stained using a Vectastain ABC-AP kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions.

Dentine Resorption Assay

Dentine resorption assays were performed according to the method of Hata et al.[13] Nonadherent bone marrow cells (1 × 10^6) in 500 μL of α-MEM containing 15% FCS were cultured in 24 well culture plates in the presence of 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ and 10% htROSCM with or without 10 ng/mL hTNF-α for 4 days. Rat primary osteoblasts were isolated by sequential digestion from the calvariae of newborn rats as described elsewhere.[47] Then, the osteoblasts (1 × 10^3 cells/well) were added to the bone marrow culture. After 4 days of coculture, cells were detached from the culture plates with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS), and were replated onto dentine slices in 96 well culture plates, which were then further incubated in α-MEM containing 15% fetal calf serum (FCS) for 6 days in the presence of 10^{-8} mol/L 1,25-(OH)$_2$D$_3$. In some experiments, the dentine resorption assay was performed using MNCs formed from nonadherent bone marrow cells by sRANKL without coculture. Nonadherent bone marrow cells were seeded on 24 well culture plates in the presence of 10^{-8} mol/L 1,25-(OH)$_2$D$_3$, 10% (vol/vol) htROSCM, and 10 ng/mL of sRANKL with or without 10 ng/mL of hTNF-α for 4 days. Cells were then trypsinized, replaced on dentine slices, and further incubated for 2 days in the presence of 100 ng/mL of sRANKL. After culture, the slices were subjected to ultrasonication to remove attached cells. Resorption pits on the slices were examined using a Jeol JSM-5200LV scanning electron microscope as previously described.[43]

[125I]-CT Binding Assay

CTRs were detected by autoradiography using [125I]-sCT as previously described.[26] Briefly, cells were rinsed once with α-MEM containing 0.1% bovine serum albumin (BSA) and incubated with 1 μCi/mL of [125I]-sCT (74 TBq/mmol) at room temperature for 2 h in the presence or absence of an excess of salmon calcitonin (sCT), (1 μg/mL). They were then rinsed three times with α-MEM containing 0.1% BSA and fixed with 50 mol/L cacodylate buffer (pH 7.3) containing 2% formaldehyde and 2% glutaraldehyde for 10 min. Finally, the cells were washed with α-MEM containing 0.1% BSA. Subsequently, the bottoms

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Table 1. Polymerase chain reaction primers used for RT-PCR analysis

Type of mRNA	Primer sequence	Nucleotide length	Sequence based on accession no. (reference)	Products (basepairs)
CTR	5'-AAGAACATGTT(C/T)CT(C/G/T)ACTTA-3'	849–868 (C1a)	L14617[16]	518
	5'-ACAAACTGGA(T/C)(T/G)CCCAGCAGGGGCAC-3'	625–644 (C1b)	L14618[16]	629
		1366–1341(C1a)		
		1288–1253(C1b)		
Cathepsin-K	5'-CAGTGTGGATCCTGTTGGG-3	445–463	AF010306	586
	5'-ACATCTTGGGGAAGCTGGC-3'	1011–1030		
GAPDH	5'-CATGGAGAAGGCTGGGGCTC-3'	306–325	X02231[52]	414
	5'-AACGGATACATTGGGGTAG-3	701–720		
TNFR type 1	5'-TGTAAGAGAGGTGGTCCCAA-3'	1188–1207	M63122	365
	5'-CACGCAGGTTCATGTCGCAA-3'	1533–1552		
TNFR type 2	5'-ATGAGAAATCCCAGGATGCAG-3'	957–977	M60469[12]	254
	5'-CTACAGACGTTCACGATGCAG-3'	1192–1212		
RANK	5'-TTAAGCCAGTGCTTCACGAC-3'	1194–1213	AF018253[37]	497
	5'-ACGTAGACCACGATGATGTCGC-3'	1669–1690		

KEY: CTR, calcitonin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RANK, receptor activator of nuclear factor κB; RT-PCR, reverse transcription-polymerase chain reaction; TNFR, tumor necrosis factor receptor.

of the wells were cut out from the culture plate, dipped in NR-M2 emulsion, and air dried. After exposure for 2–3 weeks at 4°C, autoradiographs were developed with Konicadol X and fixed with Konicafix.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Nonadherent bone marrow cells (1.1×10^7) were cultured in 60 mm tissue culture dishes (Falcon, Inc., NJ) (5.7 mL/dish) in the presence of various factors for 4 days. Total RNA was extracted using a commercial kit (Isogen, Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized from 1 μg of total RNA and subjected to PCR using an RT-PCR kit (Takara, Japan). Specific primers were used as shown in Table 1. Rat primers were used for the analysis of CTR,[16] cathepsin-K, glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[51] and TNF receptor type 1 (TNFR1). Mouse TNFR2 and human RANK primers were used for the analysis of rat TNF receptor type 2 (TNFR2) and RANK, respectively, as described elsewhere.[12,36]

PCR products were separated on a 2% agarose gel and stained with ethidium bromide. For the analysis of CTR, the PCR products were transferred to a nylon membrane (GeneScreen; NEN Research Products, Boston, MA) and subjected to southern hybridization. The rat cDNA fragment of CTR (C1a; nucleotides 849–1366) was labeled with $[\alpha\text{-}^{32}P]$-cytosine triphosphate (CTP) using a random primer DNA labeling kit (Nippon Gene) and used as a probe. Hybridization and washing were performed under stringent conditions as previously described.[38] As an internal control for RNA quantity, the same cDNA was amplified using primers specific for rat GAPDH mRNA (Table 1).[51]

Results

hTNF-α Stimulates Formation of POCs in Rat Bone Marrow Cultures

In a nonadherent rat bone marrow cell culture system that is depleted of stromal cells, the formation of TRAP-positive POCs is induced in the presence of htROSCM. hTNF-α increased the number of TRAP-positive POCs in a dose-dependent manner after 4 days of culture (Figure 1A). A concentration of 0.5 ng/mL hTNF-α significantly stimulated the formation of TRAP-

positive POCs. The same concentration of hTNF-α resulted in a similar level of stimulation when purified nonadherent bone marrow cells were used (data not shown). We also found that hTNF-α stimulated the formation of POCs that were positive for Kat1, a specific immunological marker for rat osteoclasts (Figure 1B). CTR is an another reliable marker for osteoclasts, and hTNF-α was shown to increase the number of POCs expressing CTR (Figure 1C). **Figure 2B** demonstrates CTR-positive POCs formed in the TNF-α-stimulated culture in which a number of dense grains can be seen over the cells. However, no grains were seen over the cells when an excess amount of unlabeled sCT was added to the cells (Figure 2A).

These results demonstrate that hTNF-α strongly enhances the formation of POCs, which have several osteoclastic phenotypes. Furthermore it was recently shown that mouse TNF-α, but not human TNF-α, stimulates MNC formation in a mouse bone marrow culture system in the presence of M-CSF.[22]

We then examined whether rTNF-α has activity similar to that of hTNF-α. rTNF-α has also been shown to stimulate the formation of TRAP-positive POCs (data not shown). In the presence of htROSCM, however, neither hTNF-α nor rTNF-α induced the formation of MNCs. In the absence of htROSCM, hTNF-α and rTNF-α showed no activity with regard to the formation of POCs. In this culture system, the formation of POCs and enhanced POC formation with hTNF-α were not inhibited by the addition of (100 ng/mL) OPG (data not shown).

Figure 2C shows TRAP and Kat1-positive POCs formed in the culture upon addition of 50 ng/mL of hTNF-α. Because almost all of the adherent cells were TRAP-positive or Kat1-positive, we examined the ratio of the number of TRAP-positive cells to the total number of cells in the culture. The addition of 5 and 50 ng/mL of hTNF-α increased the ratio to 62.1% and 86.2%, respectively (Table 2, top). hTNF-α also increased the ratio of the number of CTR-positive cells (Table 2, bottom).

Low Concentration of hTNF-α Augments CTR and Cathepsin-K mRNA Expression of POCs

To determine whether the stimulatory activity of hTNF-α is correlated with the expression of CTR and cathepsin-K mRNA, the level of CTR and cathepsin-K mRNA expression was analyzed by RT-PCR in cultures treated with graded concentrations of hTNF-α. The expression of CTR mRNA was markedly

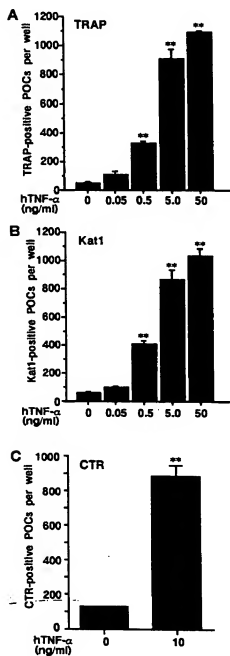Figure 1. Effect of hTNF-α on the formation of TRAP, Kat1, and CTR-positive POCs in nonadherent bone marrow cell culture. Nonadherent bone marrow cells were cultured with or without several concentrations of hTNF-α in the presence of 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ and htROSCM for 4 days. The cells were stained for TRAP (A), or immunostained with MAb Kat1 (B), as described in Materials and Methods. The cells were incubated with [^{125}I]-sCT and autoradiography was performed (C). Each value represents the mean ± SEM for four cultures. Data were analyzed by Student's t-test. **$p < 0.01$ compared with the culture without hTNF-α.
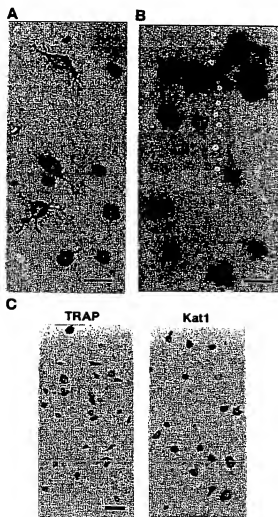

Figure 2. (A,B) Demonstration of CTR expressed in the POCs induced by TNF-α. Nonadherent bone marrow cells were cultured in the presence of htROSCM and 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ with 10 ng/mL of hTNF-α for 4 days. The cultures were then incubated with [^{125}I]-sCT for 2 h with (A) or without (B) an excess amount of unlabeled sCT followed by slight staining for TRAP. CT autoradiography was performed as described in Materials and Methods. (C) Demonstration of TRAP-positive (left panel) and Kat1-positive (right panel) POCs induced by TNF-α. Nonadherent bone marrow cells were cultured in the presence of htROSCM and 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ with 50 ng/mL of hTNF-α for 4 days. The cultures were stained for TRAP or immunostained with MAb Kat1. (A, B) Bar = 100 µm; (C) Bar = 200 µm.

increased by treatment with a low concentration (0.005 ng/mL) of hTNF-α and reached a maximal level by treatment with 0.5 ng/mL of hTNF-α (Figure 3, lanes 2 and 4). Similarly, expression of cathepsin-K, which is another marker enzyme for osteoclasts,[17,19] was markedly increased to a maximal level by treatment with low concentration (0.05 ng/mL) hTNF-α (Figure 3, lane 3).

Treatment of Culture for Forming POCs With hTNF-α Increases Resorbing Activity of MNCs Formed After Coculture With Osteoblasts

We then examined whether the POCs treated with hTNF-α had an increased ability to form bone-resorbing cells. Nonadherent bone marrow cells treated with hTNF-α (10 ng/mL) were cocultured with primary osteoblasts to form MNCs before being replated onto dentine slices. As shown in Figure 4, typical resorption pits were observed on the dentine surface by scanning electron microscopy. The resorption area was larger when the culture was treated with hTNF-α (Figure 4A) compared with the culture without hTNF-α (Figure 4B). Figure 4C provides quantitative data on dentine resorption obtained using dentine slices of exactly the same diameter. The resorption area of the MNCs was significantly increased by treatment of the culture to form

Table 2. hTNF-α increases the ratio of the number of positive cells for TRAP or CTR to the total number of cells in stroma-free bone marrow culture

Marker	hTNF-α (ng/mL)	Percentage of positive cells (%)
TRAP	0	7.90 ± 3.7
	0.05	12.3 ± 2.2
	0.5	20.7 ± 4.5[a]
	5.0	62.1 ± 6.9[a]
	50	86.2 ± 5.8[a]
CTR	0	11.2 ± 1.55
	10	60.2 ± 3.58[a]

Nonadherent cells were cultured in the presence of 10^{-8} mol/L 1.25-(OH)$_2$D$_3$ with htROSCM and several concentrations of hTNF-α for 4 days. The cells were then stained for TRAP. CTR of the cells was detected by autoradiography of bound [^{125}I]-salmon calcitonin. The ratio of the number of positive cells for TRAP or CTR to the total number of cells was analyzed. Each value represents the mean ± SEM of ten fields. Data analyzed using Student's t-test. KEY: CTR, calcitonin receptor; hTNF, human tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase.
[a] $p < 0.01$ compared with the culture without hTNF-α.

POCs with hTNF-α before the coculture with osteoblasts. These results show that treatment of nonadherent bone marrow cells with hTNF-α produces more resorbing MNCs after coculture with osteoblasts.

Effect of hTNF-α on Various Stages of POC Formation and Analysis of Several mRNAs in Bone Marrow Cells and POCs

We examined whether hTNF-α affects the early stage or the late stage of the TRAP-positive POC formation by performing temporal treatment. As shown in Figure 5, treatment of the cells
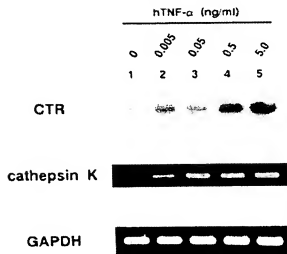

Figure 3. Demonstration of CTR and cathepsin-K mRNA expression in POCs stimulated by graded concentrations of hTNF-α. Nonadherent bone marrow cells were cultured in the presence of htROSCM and 10^{-8} mol/L 1.25-(OH)$_2$D$_3$ with 0 ng/mL (lane 1). 0.005 ng/mL (lane 2). 0.05 ng/mL (lane 3). 0.5 ng/mL (lane 4). or 5 ng/mL (lane 5) hTNF-α for 4 days. Total RNA was reverse-transcribed. and cDNA was amplified by 30 cycles of PCR for CTR, 24 cycles for cathepsin-K, or 25 cycles for GAPDH mRNA using specific primers (see Table 1). The PCR products of CTR mRNA were transferred to a nylon membrane and hybridized with [α-^{32}P]-labeled DNA fragment of rat CTR cDNA. For GAPDH and cathepsin-K mRNA analysis. the PCR products were stained with ethidium bromide.

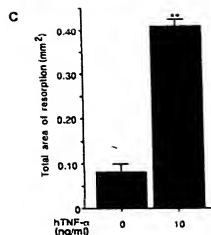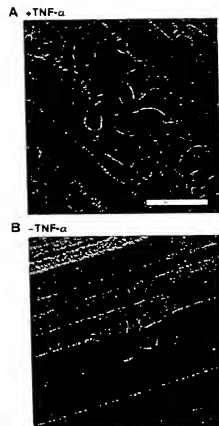Figure 4. Dentine-resorbing activity of MNCs formed in culture with and without hTNF-α. Nonadherent bone marrow cells were cultured in the presence of 1.25-(OH)$_2$D$_3$ and htROSCM with or without 10 ng/mL of hTNF-α for 4 days. Then rat primary osteoblasts were added to each well. After 4 days of coculture, cells were collected and replaced onto dentine slices, and were cultured further for 6 days in the presence of 10^{-8} mol/L 1.25-(OH)$_2$D$_3$. Resorption pits on dentine slices in culture with (A) or without (B) hTNF-α were processed for scanning microscopy (bar = 100 μm). Total resorption area was measured for each dentine slice (C). Each bar represents the mean ± SEM of quadruplicate cultures. Data analyzed by Student's t-test. $^{**}p < 0.01$ compared with the culture without hTNF-α.

with hTNF-α (10 ng/mL) on only the first or second day of culture markedly increased the formation of TRAP-positive POCs. Conversely, treatment of cells with hTNF-α on only the third or fourth day of culture did not affect the number of
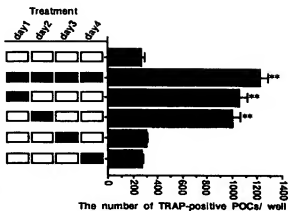
Figure 5. Time course of the effect of hTNF-α on the stimulation of TRAP-positive POC formation. Nonadherent bone marrow cells were cultured in the presence of htROSCM and 1,25-(OH)$_2$D$_3$ for 4 days with (filled bars) or without (open bars) hTNF-α (10 ng/mL) following the indicated treatment schedule. Cultures were fed and washed every day as described in *Materials and Methods*. The number of TRAP-positive mononuclear cells was counted. Each value represents the mean ± SEM of quadruplicate cultures. Data were analyzed by Student's *t*-test. **$p < 0.01$ compared with the culture without hTNF-α.

TRAP-positive POCs. These results suggest that the activity of hTNF-α to enhance POC formation is due to an ability to stimulate the early stage, but not the late stage, of formation.

To determine the population of cells responding to TNF-α, we then examined the level of mRNA of TNFR1 and TNFR2 in the cells at 0, 1, and 4 days of culture, upon stimulation with htROSCM by RT-PCR analysis using purified nonadherent bone marrow cells. As shown in **Figure 6**, bone marrow cells at day 1 of culture expressed mRNAs of TNFR1 and TNFR2, suggesting
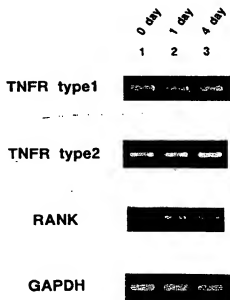

Figure 6. Expression of TNFR type 1, type 2, and RANK mRNA by bone marrow cells and POCs. Purified nonadherent bone marrow cells were collected (lane 1) and cultured in the presence of 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ and htROSCM for 1 or 4 days (lanes 2 and 3, respectively). Total RNA was reverse-transcribed, and cDNA was amplified by 30 cycles of PCR for TNFR type 1, by 32 cycles for TNFR type 2, 32 cycles for RANK, and by 25 cycles for GAPDH using specific primers (see Table 1). The PCR products were stained with ethidium bromide.
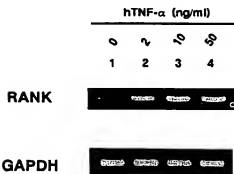

Figure 7. Treatment during the first 24 h culture with hTNF-α increased RANK mRNA expression. Purified nonadherent bone marrow cells were cultured in the presence of htROSCM and 10^{-8} mol/L 1,25-(OH)$_2$D$_3$ with 0 ng/mL (lane 1), 2 ng/mL (lane 2), 10 ng/mL (lane 3), or 50 ng/mL (lane 4) hTNF-α for 24 h. Total RNA was reverse-transcribed, and cDNA was amplified by 32 cycles of PCR for RANK, or 25 cycles for GAPDH mRNA, using specific primers (see Table 1). The PCR products were stained with ethidium bromide.

that the stimulatory effects of hTNF-α are mediated by receptors expressed on these immature bone marrow cells. We then examined whether mRNAs for RANK are expressed in these cells. Although expression of RANK mRNAs was not detected in freshly isolated nonadherent bone marrow cells, expression of RANK mRNA was detected in the cells at day 1 after 24 h of culture with htROSCM; however, no TRAP-positive adherent cells were apparent (data not shown). The culture appeared to include osteoclast precursor cells, but no POCs. We further analyzed whether treatment of bone marrow cells with TNF-α increases the expression of RANK mRNA in these precursor cells. Interestingly, the level of RANK mRNA was markedly increased by treatment at day 1, after only 24 h of culture with TNF-α (**Figure 7**).

sRANKL Inducing Formation of MNCs From POCs Stimulated With htROSCM

Because RANK mRNA was detected in cells treated with htROSCM, we then examined whether the addition of sRANKL could stimulate osteoclastogenesis in our culture system. Although sRANKL did not induce MNC formation in the absence of htROSCM, when the culture was pretreated with htROSCM for 3 days to form POCs, subsequent treatment with sRANKL induced formation of TRAP-positive MNCs (**Table 3**, top). When 100 ng/mL of hOPG was added to the culture together with sRANKL, the formation of TRAP-positive MNCs was completely inhibited. sRANKL, in combination with M-CSF, has been shown to induce MNCs from bone marrow cells.[29,55] It has also been shown, however, that this activity is dependent on the copresence of M-CSF.[22] Consistent with these earlier data, MNCs were formed when the cells were treated with M-CSF (100 ng/mL) and sRANKL (10 ng/mL) throughout the entire culture period (Table 3, bottom). In contrast, when the culture was treated separately with M-CSF (100 ng/mL) for 3 days, and then sRANKL for 2 days, TRAP-positive MNCs were not formed (Table 3, top). These results suggest that MNCs formed by the addition of sRANKL in our culture were derived from preformed POCs induced by htROSCM.

hTNF-α With sRANKL Synergistically Stimulating Formation of MNCs

In stroma-free culture, hTNF-α did not induce a significant number of TRAP-positive MNCs (**Figure 8A**). On the other

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Table 3. sRANKL induces multinucleation of POCs stimulated with h:ROSCM

Treatment			
Pretreatment (0–3 days)	sRANKL (3–5 days)	hOPG (3–5 days)	Number of TRAP⁺ MNCs/well
htROSCM	+	−	47.75 ± 4.29
htROSCM	+	+	0
M-CSF	+	−	0

Treatment	Number of TRAP⁺ MNCs/well
M-CSF + sRANKL	134 ± 10.93
htROSCM + sRANKL	75.7 ± 8.19

(Top) Purified nonadherent bone marrow cells were cultured in the presence of 1,25-(OH)$_2$D$_3$ for 3 days with htROSCM or M-CSF (100 ng/mL). Then the cultures were washed four times and incubated further for 2 days with sRANKL (10 ng/mL) in the presence or absence of hOPG (100 ng/ml). (Bottom) Purified nonadherent bone marrow cells were cultured in the presence of 1,25-(OH)$_2$D$_3$ and sRANKL (10 ng/mL) for 4 days with M-CSF (100 ng/mL) or htROSCM. Number of TRAP⁺ multinucleated cells was counted. Each value represents the mean ± SEM of quadruplicate cultures.
KEY: CSF, colony-stimulating factor; hOPG, human osteoprotegerin; M-CSF, macrophage colony-stimulating factor; MNCs, mononuclear cells; POCs, preosteoclast-like cells; sRANKL, soluble receptor activator of nuclear factor κB ligand; TRAP, tartrate-resistant acid phosphatase.

hand, sRANKL induced MNC formation in a dose-dependent manner (Figure 8A). We then examined the effect of several doses of hTNF-α on the formation of MNCs induced by various concentrations of sRANKL. Interestingly, in the presence of 10 ng/mL of sRANKL, treatment of the culture with 2–50 ng/mL of hTNF-α synergistically stimulated the formation of MNCs (Figure 8A). In contrast, in the presence of 100 ng/mL of sRANKL, hTNF-α did not stimulate formation of MNCs. MNC formation induced by sRANKL and hTNF-α was completely inhibited by the addition of 100 ng/mL of hOPG (data not shown). Figure 8B shows the typical morphology of TRAP-positive MNCs formed by 2 ng/mL of hTNF-α and 10 ng/mL of sRANKL. In Figure 8B, MNCs are large, strongly stained with TRAP, and include many nuclei. We also found that MNCs induced by sRANKL and hTNF-α efficiently resorbed dentine in the presence of sRANKL (data not shown).

Discussion

In our previous study, it was shown that POCs are mononuclear preosteoclast-like cells and are formed by the addition of htROSCM to rat stromal cell-free bone marrow cell culture. The formation of POCs was regulated by cytokines such as IL-10 and IL-15. POCs fuse to form multinucleated cells and resorb bone after coculture with primary osteoblasts. In this study we showed that human TNF-α demonstrated potent activity for stimulating the formation of POCs in rat stroma-free bone marrow culture. The POCs stimulated by TNF-α had marked expression of several osteoclastic phenotypes, including the expression of TRAP, Kat1 antigen, CTR, and cathepsin-K. Interestingly, an extremely low concentration of TNF-α (0.005 ng/ml) increased the level of CTR and cathepsin-K mRNA.

It is well known that TNF-α has the ability to stimulate bone resorption. It was also reported that the activity of TNF-α is mediated by osteoblasts.[31,50] However, because our culture was completely depleted of stromal cells, we can exclude the possibility of the indirect effect of TNF-α mediated by osteoblasts. We found that hTNF-α exerted strong activity in our stroma-free bone marrow culture by stimulating cells at a very early stage (only the first 24 h) of the culture. The activity of hTNF-α was thought to be mediated by hematopoietic osteoclast progenitor cells in our culture. In support of our data, several effects of TNF-α on the proliferation and differentiation of human hemopoietic stem cells and myeloid progenitor cells have been reported.[6,7,15,41] For example, TNF-α has shown differentiation-inducing activity on hematopoietic progenitor cells in the presence of other cytokines such as GM-CSF and IL-3. A culture of CD34⁺ hematopoietic progenitor cells (HPCs) with GM-CSF and TNF-α has been shown to result in the generation of dendritic Langerhans cells,[6] whereas TNF-α alone had no effect on HPC development. TNF-α with GM-CSF also induced differentiation into dendritic-like cells of a CD34⁺ myelomonocytic cell line (KG1).[45] These data, along with our results, suggest that hematopoietic progenitor cells can respond to TNF-α and differentiate into dendritic cells or preosteoclasts depending on factors present in the environment.

Neither hTNF-α nor rTNF-α induced MNCs in rat stroma-free bone marrow culture stimulated with htROSCM. Recently, Azuma et al.[4] and Kobayashi et al.[22] used mouse TNF-α to induce osteoclast differentiation using mouse bone marrow culture treated with M-CSF. We added various concentrations of rat TNF-α (5–500 ng/ml) with M-CSF (1–100 ng/ml) to rat stroma-free bone marrow cells. We also pretreated rat bone marrow cells

A

Number of TRAP-positive MNCs

hTNF-α (ng/ml): 0 2 10 50   0 2 10 50   0 2 10 50   0 2 10 50
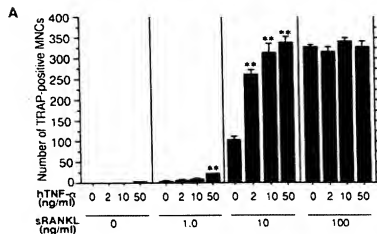sRANKL (ng/ml):   0   1.0   10   100

B

Figure 8. Cooperative effect of hTNF-α and sRANKL on the formation of TRAP-positive MNCs. (A) Purified nonadherent bone marrow cells were cultured in the presence of 1,25-(OH)$_2$D$_3$ and htROSCM and various concentrations of hTNF-α and/or sRANKL for 4 days. At the end of the culture, cells were fixed and stained for TRAP. The number of TRAP-positive MNCs was counted in each well. Each bar represents the mean ± SEM of quadruplicate cultures. Data analyzed by Student's t-test. **p < 0.01 compared with the culture with sRANKL alone. (B) Morphology of TRAP-positive MNCs formed in the culture with 2 ng/mL of hTNF-α in the presence of sRANKL (10 ng/mL). Bar = 200 μm.

with M-CSF (100 ng/mL) for 3 days and then added various concentrations of rat TNF-α. Nevertheless, rat TNF-α failed to induce MNC formation in the rat bone marrow culture system in the presence of M-CSF, although the experiments were repeated many times (data not shown). The reason why we could not induce osteoclasts with rat TNF-α and M-CSF remains unknown. However, rat bone marrow cells treated with high concentrations of M-CSF, which were large adherent cells that included macrophage polykaryons, appeared to be different from those in mouse bone marrow culture (data not shown).

It is known that the extracellular domain of TNFR1 is the most conserved among species, whereas that of TNFR2 is species-specific.[32,49] Kobayashi et al.[22] also demonstrated that mouse and human TNF-α had different levels of activity in mouse osteoclast formation. They claimed that because both TNFR1 and TNFR2 are necessary for osteoclast formation, and human TNF-α binds preferentially to mouse TNFR1 rather than mouse TNFR2, the potency of human TNF-α to induce osteoclast formation is much weaker than that of mouse TNF-α. As to the activity of POC formation, we did not see differences between rat and human TNF-α. Although the possible difference in affinity between human and rat TNF-α for rat TNFR1 and TNFR2 was not clarified, a signal through TNFR1 may be enough to stimulate POC formation. Thus, the signals essential for POC formation may be different from those for MNC formation. In addition, there are other differences between the two culture systems. First, the type of cells responding to TNF-α appears to be different. The target cells for TNF-α in their culture system appear to be macrophages. In contrast, the TNF-α-responsive cells in our rat bone marrow culture system appear to be hematopoietic progenitor cells. Second, sensitivity to the concentration of TNF-α was different. We found that POC formation was stimulated even at a low concentration (0.5 ng/mL) of TNF-α. Conversely, they used high concentrations of TNF-α (10–100 ng/ml) to induce MNC formation.[4,22] Pfeilschifter et al.[40] and van der Pluijn et al.[52] also reported that relatively low concentrations of TNF-α stimulated osteoclast formation in human bone marrow culture and fetal mouse metacarpals, respectively. The sensitivity of macrophage and hematopoietic immature cells to TNF-α may be different. Third, in our culture, TNF-α alone had no influence on the development of cells with osteoclastic phenotypes, and cooperation with some factors in htROSCM was necessary to enhance the activity of TNF-α. However, we have obtained evidence that htROSCM does not contain M-CSF. In a previous study, we found that the activity of M-CSF in htROSCM was destroyed, and the addition of M-CSF did not induce POCs in rat stroma-free bone marrow culture.[25] As stated earlier, TNF-α with M-CSF did not induce MNCs and POCs in the absence of htROSCM in our culture. In addition, the coprescence of M-CSF, but not htROSCM, is required for MNC formation by the addition of sRANKL. Therefore, the possibility that htROSCM contains M-CSF can be excluded. The activity of htROSCM is also different from that of RANKL, because it is not blocked by the addition of hOPG. The factor that induced formation of POCs, but not MNCs, has not been well elucidated. Further biochemical analysis of this activity in htROSCM is necessary and is currently in progress in our laboratory.

Interestingly, treatment with hTNF-α synergistically stimulated the formation of TRAP-positive MNCs induced by 10 ng/mL of sRANKL. This result raises some questions as to how TNF-α stimulates osteoclast formation in the presence of RANKL. We found that treatment of the culture with hTNF-α increased RANK mRNA expression, which may result in efficient fusion of POCs by stimulation with RANKL. On the other hand, in the presence of a high concentration of RANKL (100

ng/mL), hTNF-α did not stimulate MNC formation, suggesting that the level of RANK mRNA expression is maximal and may not be enhanced. Alternatively, one of the molecular mechanisms underlying the synergistic action may involve cooperative activation of transcription factors.[39] NF-κB has been proposed to be involved in osteoclast differentiation as a common signaling molecule.[10,18] However, another unknown signaling transcription factor induced by TNF-α or RANKL may activate genes that are necessary for multinucleation of POCs. Alternatively, crosstalk between signal transduction pathways upstream of activation of the transcription factor may contribute to the synergistic effect.[39] Further investigation is necessary to elucidate the mechanism of the synergistic effect of TNF-α and sRANKL.

The role of TNF-α in rheumatoid arthritis (RA) has been studied.[9,24,42] In addition to the accumulation of TNF-α in synovial fluids from rheumatic patients,[42] it has been reported that transgenic mice expressing human TNF-α experience erosive arthritis.[20] Moreover, clinical trials of RA with anti-TNF-α antibody and TNFR-Fc fusion protein have revealed a marked benefit in all assessable aspects of the disease.[9] Recently, Takayanagi et al.[48] and Gravallese et al.[11] reported that RANKL is expressed in fibroblasts of synovial tissue in RA patients. These results, combined with our findings, suggest that cooperation of TNF-α and RANKL plays an important role in bone destruction in inflammatory diseases such as RA.

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